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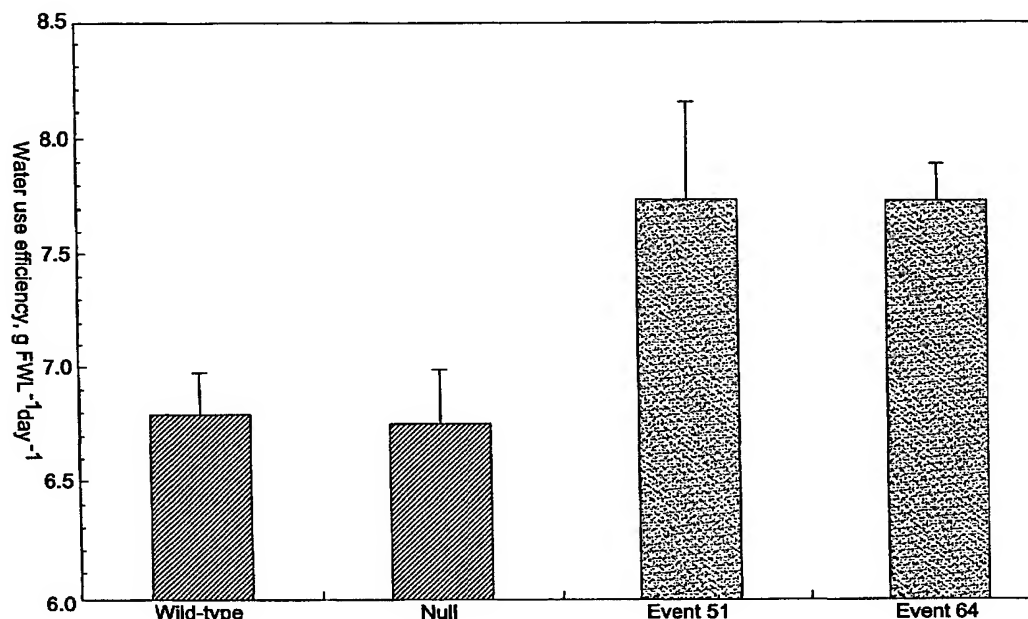
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(54) Title: METHODS FOR MODULATING WATER-USE EFFICIENCY OR PRODUCTIVITY IN A PLANT



(57) Abstract: A method for modulating water-use efficiency or productivity in a plant is provided. In particular, stomatal aperture in a plant can be regulated by transforming the plant with a polynucleotide capable of modifying malate accumulation in the plant.



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## METHODS FOR MODULATING WATER-USE EFFICIENCY OR PRODUCTIVITY IN A PLANT

### FIELD OF THE INVENTION

The invention is drawn to genetic engineering of plants to improve agronomic performance, particularly for increasing water-use efficiency in plants.

### 5 BACKGROUND OF THE INVENTION

Plant growth is often limited by the availability of water. In plants, the majority of all water loss occurs through pores on the leaf surface, which are called stomata. The sizes of the stomatal pores in a leaf are variable and control the rate of diffusion of water vapor out of the plant. In addition to controlling water loss,  
10 stomata allow CO<sub>2</sub> to diffuse into the leaf for photosynthesis. This common pathway for gas exchange between the leaf and the atmosphere results in a large amount of water vapor escaping from the plant during the influx of CO<sub>2</sub>. However, stomatal aperture is typically substantially greater than that needed for maintaining maximal photosynthetic rates.

15 Each stomate is formed by two guard cells. Together, the two guard cells form a stomatal pore. Opening and closing of stomata are caused by specialized biochemical processes in the guard cells that occur in response to environmental changes. The pore is opened by an increase in osmotic pressure in the guard cells, which is the result of the uptake and synthesis of osmotically active compounds  
20 and a corresponding uptake of water. The increase in guard-cell volume causes the pore to open.

To minimize water loss from leaves, stomatal aperture is regulated. During water shortage, plants prevent dehydration by closing their stomata partially or completely. When water is not limiting and other environmental conditions favor  
25 photosynthesis and plant growth, stomata are open, allowing CO<sub>2</sub> to enter for photosynthesis. Because plant growth is often limited by the availability of water and because the amount of water loss greatly exceeds CO<sub>2</sub> uptake necessary for

photosynthetic carbon reduction, molecular mechanisms are needed to increase water-use efficiency in plants.

### SUMMARY OF THE INVENTION

5           An object of the present invention is to provide methods and compositions for improving water-use efficiency in plants.

          Another object of the present invention is to provide methods and compositions for increasing drought tolerance in plants.

          Another object of the present invention is to provide methods and  
10       compositions for increasing irrigation efficiency.

          Another object of the present invention is to provide methods and compositions for increasing productivity under conditions when water is not limiting.

          Another object of the present invention is to provide methods and  
15       compositions for increasing heat tolerance in plants.

          The methods comprise engineering a plant to modify malate accumulation in the plant to alter stomatal conductance to water vapor. Polynucleotides capable of modifying the accumulation of malate in the plant can be used in cassettes or constructs for expression in plants or plant cells of interest. Transformed plants,  
20       tissues, and seeds having improved water-use efficiency are provided.

          Methods for modulating stomatal aperture or altering water-use efficiency in a plant are provided, the methods comprise:

- a)       transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the  
25       polynucleotide is capable of modulating malate accumulation in the plant cell, with the proviso that when the polynucleotide encodes phosphoenolpyruvate carboxylase, the plant is other than potato or tobacco;
- b)       regenerating plants from the transformed cell; and
- 30       c)       selecting for plants exhibiting an altered stomatal aperture or altered water-use efficiency or characteristic correlated to same.

Also provided are methods for increasing productivity in a plant comprising:

- a) transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the polynucleotide is capable of modulating malate accumulation in the plant cell, with the proviso that when the polynucleotide encodes phosphoenolpyruvate carboxylase, the plant is other than potato or tobacco;
- b) regenerating plants from the transformed cell; and
- c) selecting for regenerated plants exhibiting improved productivity.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1: Gene construct used in transformation examples.

Figure 2A: Northern blots depicting maize malic enzyme mRNA for wild-type and transformed tobacco leaves.

Figure 2B: Malic enzyme activity in wild-type and transformed tobacco leaves.

Figure 3: Malic enzyme activity in wild-type and transformed tobacco leaves.

Figure 4: Malate content in wild-type and transformed tobacco leaves.

Figure 5: Steady-state stomatal conductance in wild-type and transformed tobacco leaves.

Figure 6: Water-use efficiency in wild-type and transformed tobacco plants.

Figure 7: Drought response of wild-type and transformed tobacco plants.

#### DETAILED DESCRIPTION OF THE INVENTION

The present invention is drawn to compositions and methods for modulating stomatal aperture in plants. In particular, methods of the invention are useful for increasing water-use efficiency in plants. Methods encompass transforming a plant with a polynucleotide capable of modulating malate accumulation in the plant. Typical enzymes that can modulate the level of malate include malic enzyme, malate dehydrogenase, phosphoenolpyruvate carboxylase,

glycolytic enzymes, or starch degradation enzymes. As used herein, “a polynucleotide corresponding to” a particular enzyme means the polynucleotide is capable of altering the expression of the enzyme. The altered malate concentration leads to a change in guard-cell volume that alters stomatal pore size.

5 Polynucleotides useful in the invention encompass antisense or coding constructs for the enzymes of the invention. Thus, antisense sequences can be used to decrease expression of the enzyme. Likewise, coding sequences can be utilized to increase or decrease expression. Also single chain antibodies can be used to modulate the level of the enzymes.

10 As noted above, methods for modulating the stomatal aperture are provided. The benefits of modifying stomatal aperture are significant. For example, decreasing stomatal aperture is expected to improve drought tolerance. As water is a limited resource in many areas of the world, drought tolerance is a valuable trait. In the case of a well-watered crop, increasing the stomatal aperture  
15 is expected to improve heat tolerance and productivity.

Methods of the invention comprise expressing a polynucleotide capable of modulating the level of malate in a plant. A broad range of enzymes can influence the level of malate. Such enzymes include but are not limited NADP-malic enzyme (ME), malate dehydrogenase, phosphoenolpyruvate carboxylase, and  
20 enzymes of the pathway that convert starch to malate, e.g., glycolytic and starch degrading enzymes. Thus, a plant may be transformed with one or a combination of the enzymes. Other polynucleotides capable of modulating the level of malate in a plant include those encoding malate or dicarboxylic transporter.

The nucleotide sequences encoding ME are known in the art. For example,  
25 C<sub>4</sub> NADP<sup>+</sup>-ME full-length cDNAs have been cloned from several monocot and dicot species (e.g., AC# J05130, AC# J03825). There is NAD<sup>+</sup> malic enzyme as well. Additionally, other enzymes of interest are known and include malate dehydrogenase, phosphoenolpyruvate carboxylase, glycolytic enzymes such as, hexokinase, hexosephosphate isomerase, phosphofructokinase, aldolase,  
30 fructokinase, triose phosphate isomerase, glyceraldehyde 3-phosphate dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, enolase, pyruvate kinase, and starch degrading enzymes such as  $\alpha$ -amylase,  $\beta$ -amylase, starch phosphorylase, D enzyme,  $\alpha$ -glucosidase, debranching enzyme, and  $\alpha$ -1,4-

glucan lyase. Examples of suitable polynucleotides encoding the enzymes can be found in the following cites: phosphofructokinase, *J. Biol. Chem.* 265(30):18366-18371 (1990); the complete nucleotide sequence of cDNA encoding phosphoenolpyruvate carboxylase from cultured tobacco, *Plant Mol. Biol.* 17(3):535-540 (1991); triose-phosphate isomerase, *Mol. Biol. Evolution* 7(1):74-81 (1990); glyceraldehyde 3-phosphate dehydrogenase, *Plant Physiology (Rockville)* 105(1):357-367 (1994); pyruvate kinase, *Plant Physiology (Bethesda)* 96(4):1283-1288 (1991); fructokinase, *Plant Physiology (Bethesda)* 93(1):353-355 (1990); aldolase, *Plant Mol. Biol.* 21(2):331-340 (1993); NADP<sup>+</sup>-malate dehydrogenase, *Proceedings of the National Academy of Sciences of the United States of America* 92(12):5620-5624 (1995); enolase, *Plant Cell* 3(7):719-736 (1991); barley alpha-amylase, *Applied Microbiology and Biotechnology* 49(4):385-392 (1998); B-amylase, beta-amylase gene of *Arabidopsis thaliana*, *Plant Physiology (Rockville)* 114(2):575-582 (1997); starch phosphorylase, sequencing of the phosphorylase cDNA, *Plant Cell* 1(5):559-566 (1989); D-enzyme, from potato tubers and its activity characterized, *J. Biol. Chem.* 268(2):1391-1396 (1993); alpha-glucosidase, *Bulletin of the Research Institute for Bioresources Okayama Univ.* 5(1):1-9 (1997); a-Glucosidase, *Bioscience Biotech. and Biochem.* 57(11):1902-1905 (1993); starch debranching enzyme from developing rice endosperm: Purification, cDNA and chromosomal localization of the gene, *Planta (Heidelberg)* 199(2):209-218 (1996).

It is recognized that the sequences disclosed above may be used as well as variants or fragments thereof. Additionally, polynucleotides encoding variant proteins may be utilized. It is only important that the polynucleotide modulates the accumulation of malate in the plant, preferably in the leaves, and more preferably in the guard cells.

It is recognized that with these nucleotide sequences, antisense constructions, cosuppression or other method for down-regulation complementary to at least a portion of the messenger RNA (mRNA) for the target enzymes can be constructed. Antisense nucleotides are constructed to hybridize with the corresponding mRNA. Therefore, modifications of the sequences may be made as long as the sequences hybridize to and interfere with expression of the corresponding mRNA. In this manner, antisense constructions having 70%,

preferably 80%, more preferably 85% sequence similarity to the corresponding antisense sequences may be used. Furthermore, portions of the antisense nucleotides may be used to disrupt the expression of the target gene. Generally, sequences of at least about 20, 30, 50 nucleotides, 100 nucleotides, 200

5 nucleotides, or greater may be used.

While the methods can be used in any plant the following crops are of particular interest, but are not limited to maize, sorghum, soybean, sunflower, safflower, alfalfa, canola, tomato, wheat, rice, peanut, and cotton.

The sequences to be introduced may be used in expression cassettes for  
10 expression in any plant of interest where expression in the plant is necessary for transcription. While it may be preferable to express the sequences using heterologous promoters, the native promoter sequences may be used. Such constructs would change expression levels of the enzymes of interest in the plant or plant cell. Thus, the phenotype of the plant or plant cell is altered.

15 Where expression cassettes are needed, such expression cassettes will comprise a transcriptional initiation region linked to the coding sequence or antisense sequence of the nucleotides of interest. Such an expression cassette is provided with a plurality of restriction sites for insertion of the sequence to be under the transcriptional regulation of the regulatory regions. The expression  
20 cassette may additionally contain selectable marker genes.

The transcriptional initiation region, the promoter, may be native or analogous or foreign or heterologous to the plant host. Additionally, the promoter may be a natural sequence or alternatively a synthetic sequence. By foreign is intended that the transcriptional initiation region is not found in the native plant  
25 into which the transcriptional initiation region is introduced. As used herein a chimeric gene comprises a coding sequence operably linked to a transcription initiation region that is heterologous to the coding sequence.

The transcriptional cassette will include in the 5'-to-3' direction of transcription, a transcriptional and translational initiation region, a polynucleotide  
30 of interest, and a transcriptional and translational termination region functional in plants. The termination region may be native with the transcriptional initiation region, may be native with the polynucleotide of interest, or may be derived from another source. Convenient termination regions are available from the Ti-plasmid



of *A. tumefaciens*, such as the octopine synthase and nopaline synthase termination regions. See also Guerineau *et al.* (1991) *Mol. Gen. Genet.* 262:141-144; Proudfoot (1991) *Cell* 64:671-674; Sanfacon *et al.* (1991) *Genes Dev.* 5:141-149; Mogen *et al.* (1990) *Plant Cell.* 2:1261-1272; Munroe *et al.* (1990) *Gene* 91:151-158; Ballas *et al.* (1989) *Nucleic Acids Res.* 17:7891-7903; Joshi *et al.* (1987) *Nucleic Acids Res.* 15:9627-9639. Additionally the 3' termination sequence (e.g., nopaline synthase or CaMV) can be used. The cassette may also contain at least one additional sequence to be co-transformed into the organism. Alternatively, the additional sequence(s) can be provided on another expression cassette.

10 In preparing the expression cassette, the various polynucleotide fragments may be manipulated, so as to provide for the polynucleotide of interest in the proper orientation and, as appropriate, in the proper reading frame. Toward this end, adapters or linkers may be employed to join the polynucleotide fragments or other manipulations may be involved to provide for convenient restriction sites, removal of superfluous sequences, removal of restriction sites, or the like. For this purpose, *in vitro* mutagenesis, primer repair, restriction, annealing, resubstitutions, e.g., transitions and transversions, may be involved.

A variety of promoters may be used in the practice of the invention. In particular, PCaMV35S plus enhancer (strong and constitutive), PrbcS (strong, leaf preferred), Pcab (strong, leaf preferred) and Pnos (weak and constitutive) can be used. Other promoters of interest include, the core promoter of the Rsyn7 (copending U.S. Patent Application Serial No. 08/661,601), the core CaMV 35S promoter (Odell *et al.* (1985) *Nature* 313:810-812); rice actin (McElroy *et al.* (1990) *Plant Cell* 2:163-171); ubiquitin (Christensen *et al.* (1989) *Plant Mol. Biol.* 12:619-632 and Christensen *et al.* (1992) *Plant Mol. Biol.* 18:675-689); pEMU (Last *et al.* (1991) *Theor. Appl. Genet.* 81:581-588); MAS (Velten *et al.* (1984) *EMBO J.* 3:2723-2730); ALS promoter (U.S. Patent Application Serial No. 08/409,297), and the like. Other constitutive promoters include, for example, U.S. Patent Nos. 5,608,149; 5,608,144; 5,604,121; 5,569,597; 5,466,785; 5,399,680; 5,268,463; and 5,608,142. Preferably guard cell or epidermal cell promoters, such as KAT1 (Nakamura *et al.* (1995) *Plant Physiology* 109:371-374, kin1, cor6.6 (Wang and Cutler (1995) *Plant Mol. Biol.* 28:619-634), the 0.3-kb 5' promoter fragment of ADPase (Müller-Röber *et al.* (1994) *Plant Cell* 6:601-612), Rha1

(Terry *et al.* (1993) *Plant Cell* 5:1761-1769) are used. The use of drought inducible, guard-cell promoters, such as CdeT6-19 (Taylor *et al.* (1995) *Plant Journal* 7:129-134) would also be desirable.

The genes of interest of the present invention can be targeted to the chloroplast for expression. In this manner, where the gene of interest is not directly inserted into the chloroplast, the expression cassette will additionally contain a gene encoding a transit peptide to direct the gene of interest to the chloroplasts. Such transit peptides are known in the art. See, for example, Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; and, Shah *et al.* (1986) *Science* 233:478-481. Chloroplast targeting sequences additionally include the chloroplast small subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco) (de Castro Silva Filho *et al.* (1996) *Plant Mol. Biol.* 30:769-780; Schnell, *et al.* (1991) *J. Biol. Chem.* 266(5):3335-3342); 5-(enolpyruvyl)shikimate-3-phosphate synthase (EPSPS) (Archer *et al.* (1990) *J. Bioenerg. Biomemb.* 22(6):789-810); tryptophan synthase (Zhao *et al.* (1995) *J. Biol. Chem.* 270(11):6081-6087); plastocyanin (Lawrence *et al.* (1997) *J. Biol. Chem.* 272(33):20357-20363); chorismate synthase (Schmidt *et al.* (1993) *J. Biol. Chem.* 268(36):27477-27457); and the light harvesting chlorophyll a/b binding protein (LHBP) (Lamppa *et al.* (1988) *J. Biol. Chem.* 263:14996-14999). See also Von Heijne *et al.* (1991) *Plant Mol. Biol. Rep.* 9:104-126; Clark *et al.* (1989) *J. Biol. Chem.* 264:17544-17550; della-Cioppa *et al.* (1987) *Plant Physiol.* 84:965-968; Romer *et al.* (1993) *Biochem. Biophys. Res. Commun.* 196:1414-1421; and Shah *et al.* (1986) *Science* 233:478-481. Additionally, the *rbcS* transit sequence can be used for targeting to the chloroplast stroma.

It is possible that direct chloroplast transformation could be used for expression of the enzymes of interest. Methods for transformation of chloroplasts are known in the art. See, for example, Svab *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; Svab and Maliga (1993) *Proc. Natl. Acad. Sci. USA* 90:913-917; Staub and Maliga (1993) *Embo J.* 12:601-606. The method relies on particle gun delivery of the polynucleotide of interest containing a selectable marker and targeting of the polynucleotide of interest to the plastid genome through

homologous recombination. Additionally, plastid transformation can be accomplished by transactivation of a silent plastid-borne transgene by tissue-specific expression of a nuclear-encoded and plastid-directed RNA polymerase. Such a system has been reported in McBride *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:7301-7305.

The sequences of the present invention can be used to transform or transfect any plant. In this manner, genetically modified plants, plant cells, plant tissue, seed, and the like can be obtained. Transformation protocols as well as protocols for introducing nucleotide sequences into plants may vary depending on the type of plant or plant cell, *i.e.* monocot or dicot, targeted for transformation. Suitable methods of introducing nucleotide sequences into plant cells and subsequent insertion into the plant genome include microinjection (Crossway *et al.* (1986) *Biotechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium*-mediated transformation (US Pat. No. 5,563,055), direct gene transfer (Paszkowski *et al.* (1984) *EMBO J.* 3:2717-2722), and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent No. 4,945,050; Tomes *et al.* (1995) "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg and Phillips (Springer-Verlag, Berlin); and McCabe *et al.* (1988) *Biotechnology* 6:923-926). Also see Weissinger *et al.* (1988) *Annual Rev. Genet.* 22:421-477; Sanford *et al.* (1987) *Particulate Science and Technology* 5:27-37 (onion); Christou *et al.* (1988) *Plant Physiol.* 87:671-674 (soybean); McCabe *et al.* (1988) *Bio/Technology* 6:923-926 (soybean); Finer and McMullen (1991) *In Vitro Cell Dev. Biol.* 27P:175-182 (soybean); Singh *et al.* (1998) *Theor. Appl. Genet.* 96:319-324 (soybean); Datta *et al.* (1990) *Biotechnology* 8:736-740 (rice); Klein *et al.* (1988) *Proc. Natl. Acad. Sci. USA* 85:4305-4309 (maize); Klein *et al.* (1988) *Biotechnology* 6:559-563 (maize); Tomes *et al.* "Direct DNA Transfer into Intact Plant Cells via Microprojectile Bombardment," in *Plant Cell, Tissue, and Organ Culture: Fundamental Methods*, ed. Gamborg (Springer-Verlag, Berlin) (1995) (maize); Klein *et al.* (1988) *Plant Physiol.* 91:440-444 (maize); Fromm *et al.* (1990) *Biotechnology* 8:833-839 (maize); Hooydaas-Van Slogteren and Hooykaas (1984) *Nature (London)* 311:763-764; Bytebier *et al.* (1987) *Proc. Natl. Acad. Sci. USA* 84:5345-5349 (Liliaceae);

De Wet *et al.* (1985) in *The Experimental Manipulation of Ovule Tissues*, ed. Chapman *et al.* (Longman, New York), pp. 197-209 (pollen); Kaeppler *et al.* (1990) *Plant Cell Reports* 9:415-418; and Kaeppler *et al.* (1992) *Theor. Appl. Genet.* 84:560-566 (whisker-mediated transformation); D'Halluin *et al.* (1992) *Plant Cell* 4:1495-1505 (electroporation); Li *et al.* (1993) *Plant Cell Reports* 12:250-255 and Christou and Ford (1995) *Annals of Botany* 75:407-413 (rice); Osjoda *et al.* (1996) *Nature Biotechnology* 14:745-750 (maize via *Agrobacterium tumefaciens*); all of which are herein incorporated by reference.

The modified plant may be grown into plants in accordance with conventional ways. See, for example, McCormick *et al.* (1986) *Plant Cell. Reports* 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

In practicing the present invention, the malate content generally will be modulated by at least about 5%, frequently at least about 10%, preferably at least about 15 %, more preferably in the range of from about 15% to about 65%, and most preferably in the range of from about 15% to about 45%.

Using the methods of the present invention, the stomatal conductance is modulated at least about 5%, preferably at least about 10%, more preferably in the range of from about 15% to about 80%, and most preferably in the range of from about 15% to about 65%. As indicated above, the stomatal conductance will be reduced where increased water efficiency is desired. In order to increase heat stress tolerance, stomatal conductance will be increased.

Generally the present methods can be employed to increase water-use efficiency by at least about 5%, preferably at least about 10% and more preferably in the range of from 10% to 20%.

The following examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

A cDNA sequence encoding C<sub>4</sub> NADP<sup>+</sup>-malic enzyme (ME) from maize (AC# J05130) was fused to a cDNA sequence encoding an rbcS chloroplast transit peptide from petunia (Dean *et al.* (1987) *Nucleic Acids Res.* 15:4655-4668), a modified mas promoter (Ni *et al.* (1995) *Plant Journal* 7:661-676), and a pinII terminator (An *et al.* (1989) *Plant Cell* 1:115-122; Figure 1). The modified mas promoter directs expression in a variety of plant tissues, including leaf epidermal cells and guard cells. The ME gene construct was linked to the nptII gene, which confers kanamycin resistance for use as a selectable marker (Herrera-Estrella *et al.* (1983) *EMBO J.* 2:987-995). The expression cassette was cloned into the pBK-CMV expression vector (Stratagene, Inc.).

Gene constructs were introduced into tobacco by *Agrobacterium*-mediated transformation (Horsch *et al.* (1985) *Science* 227:1229-1231; An *et al.* (1986) *Plant Physiol.* 81:301-305). More than 10 kanamycin resistant transformation events were regenerated.

Maize ME mRNA transcript accumulated to high levels in the transgenic events, as demonstrated by the representative northern blot shown in Figure 2A. A maize ME gene specific probe was used to assay for the presence of mRNA encoding maize ME. Total extractable ME activity was increased as much as 30 fold in the ME-transformed events relative to wild-type tobacco (Figure 2B). Malic enzyme activity was assayed as described by Kanai and Edwards (1973) *Plant Physiol.* 51:1133-1137. The selfed progeny of the five events depicted in Figure 3 were used for phenotypic analysis. Homozygous individuals from these events had ME activity ranging from 5 to 18 times that of wild-type tobacco in young leaves. Individuals containing one or two copies of the ME gene as well as null segregants derived from these events were analyzed after two generations of selfing (T<sub>2</sub> generation). All data depicted in the figures are from individuals with two copies of the gene except as noted.

Whole-leaf malate content was decreased by 23 to 61% in the ME-transformed plants relative to wild-type and null segregants (Figure 4). Whole-leaf malate content was assayed as described by Ku *et al.* (1981) *Plant Physiol.* 68:1073-1080. It is likely that this decrease in whole-leaf malate is correlated with

a decrease in guard-cell malate because the modified mas promoter is known to express in both guard and epidermal cells (Ni *et al.* (1995) *Plant Journal* 7:661-676).

Steady-state stomatal conductance to water vapor was significantly  
5 negatively correlated with total extractable ME activity in a segregating population that included individuals derived from all five transgenic events and wild-type ( $F_{1,9}=1.49$ ;  $P=0.013$ ; Figure 5). Stomatal conductance was measured in attached, fully expanded leaves using a LiCor 6400 Portable Photosynthesis System equipped with the 6400-02B LED light source (LiCor, Inc., Lincoln Nebraska).  
10 Measurement of steady-state stomatal conductance was performed by continuously monitoring stomatal conductance under  $1200 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  of light,  $25^{\circ}\text{C}$ , and constant relative humidity. After 30 minutes of steady state conductance, the conductance value was recorded.

Water-use efficiency was significantly increased in the ME-transformed  
15 plants relative to null segregants and wild-type tobacco (Figure 6). Neither the rate of growth nor the root-shoot ratio differed significantly among the ME-transformants, null segregants or wild-type under these conditions (data not shown). The plants were grown in  $\frac{1}{2}$  strength Hoagland's solution in a Conviron growth chamber equipped with incandescent and florescent lights that provided  
20  $400 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$  at plant level,  $25/20^{\circ}\text{C}$ , and 60% relative humidity. The volume of the solution was measured and the plants were gently patted dry and weighed daily.

Under conditions of limited water availability, the wild-type and null segregants lost soil moisture more rapidly than the ME-transformed plants (Figure  
25 7). The plants were grown in 1 L pots and watered twice daily from below for 15 minutes per watering. Percent soil moisture was calculated as described by Pei *et al.* (1998) *Science* 282:287-290. Under these conditions, wilting of the ME-transformed plants was delayed relative to wild-type or null segregants (data not shown).

30 All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the

same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be  
5 obvious that certain changes and modifications may be practiced within the scope of the appended claims.

## THAT WHICH IS CLAIMED:

1. A method for modulating stomatal aperture in a plant comprising:
  - a) transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the polynucleotide is capable of modulating malate accumulation in the plant cell, with the proviso that when the polynucleotide encodes phosphoenolpyruvate carboxylase, the plant is other than potato or tobacco;
  - b) regenerating plants from the transformed cell; and
  - c) selecting for regenerated plants exhibiting an altered stomatal aperture or altered water-use efficiency or characteristic correlated to same.
2. The method of claim 1, wherein the malate level is decreased.
3. The method of claim 1, wherein the malate level is increased.
4. The method of claim 1, wherein the polynucleotide corresponds to malic enzyme.
5. The method of claim 1, wherein the polynucleotide is expressed in leaves of the plant.
6. The method of claim 5, wherein the polynucleotide is expressed in guard cells of the plant.
7. The method of claim 1, wherein the construct further comprises a DNA sequence encoding a transit peptide.
8. The method of claim 7, wherein the transit peptide is a chloroplast transit sequence.



9. The method of claim 1, wherein the plant is selected from the group consisting of maize, soybean, sorghum, sunflower, alfalfa, canola, safflower, tomato, wheat, rice, peanut, and cotton.

- 5           10. A method for modulating water-use efficiency in a plant comprising;
- 10           a) transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the polynucleotide is capable of modulating malate accumulation in the plant cell, with the proviso that when the polynucleotide corresponds to phosphoenolpyruvate carboxylase, the plant is other than potato or tobacco;
- 15           b) regenerating plants from the transformed cell; and
- c) selecting for regenerated plants exhibiting an altered stomatal aperture or altered water-use efficiency or characteristic correlated to same.

11. The method of claim 10, wherein the malate level is decreased.

20           12. The method of claim 10, wherein the malate level is increased.

13. The method of claim 10, wherein the polynucleotide corresponds to malic enzyme.

25           14. The method of claim 10, wherein the polynucleotide is expressed in leaves of the plant.

15           15. The method of claim 10, wherein the polynucleotide is expressed in guard cells of the plant.

30

16. The method of claim 10, wherein the construct further comprises a DNA sequence encoding a transit peptide.

17. The method of claim 16, wherein the transit peptide is a chloroplast transit sequence.

18. The method of claim 10, wherein the plant is selected from the  
5 group consisting of maize, sorghum, soybean, sunflower, alfalfa, canola, safflower, tomato, wheat, rice, peanut, and cotton.

19. A transformed plant comprising an isolated polynucleotide stably integrated into the plant genome, wherein the polynucleotide is operably linked to  
10 a promoter that drives expression in the plant, wherein the polynucleotide is capable of modulating the level of malate in the plant or a plant part, wherein the plant exhibits altered stomatal aperture or altered water-use efficiency or characteristic correlated to same, with the proviso that when the polynucleotide corresponds to phosphoenolpyruvate carboxylase, the plant is other than potato or  
15 tobacco.

20. The plant of claim 19, wherein the malate level is decreased.

21. The plant of claim 19, wherein the malate level is increased.  
20

22. The plant of claim 19, wherein the malate level is modulated by at least about 5%.

23. The plant of claim 22, wherein the malate level is modulated by at least about 10%.  
25

24. The plant of claim 23, wherein the malate level is modulated in the range of from about 15% to about 65%.

25. The plant of claim 19, wherein stomatal conductance is modulated at least about 5%.  
30

26. The plant of claim 25, wherein stomatal conductance is modulated at least about 10%.

27. The plant of claim 26, wherein stomatal conductance is modulated  
5 in the range of from about 15% to about 80%.

28. The plant of claim 27, wherein stomatal conductance is modulated in the range of from about 15% to about 65%.

10 29. The plant of claim 19, wherein water-use efficiency is increased by at least about 5%.

30. The plant of claim 29, wherein water-use efficiency is increased by at least about 10%.

15

31. The plant of claim 30, wherein water-use efficiency is increased in the range of from about 10% to about 20%.

32. The plant of claim 19, wherein the polynucleotide corresponds to  
20 malic enzyme.

33. The plant of claim 19, wherein the construct further comprises a DNA sequence encoding a transit peptide.

25 34. The method of claim 33, wherein the transit peptide is a chloroplast transit peptide.

35. The plant of claim 19, wherein the plant is selected from the group consisting of maize, sorghum, soybean, sunflower, safflower, alfalfa, canola,  
30 tomato, wheat, rice, peanut, and cotton.

36. A plant cell comprising a polynucleotide capable of modulating the level of malate in the plant or plant part, wherein a plant regenerated from the plant cell exhibits altered stomatal aperture or altered water-use efficiency.
- 5           37. The plant cell of claim 36, wherein the malate level is decreased.
38. The plant cell of claim 36, wherein the malate level is increased.
39. A method for modulating stomatal aperture in a plant comprising:
- 10           a) transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the polynucleotide is capable of modulating malate accumulation in the plant cell, and wherein the polynucleotide encodes an enzyme other than phosphoenolpyruvate carboxylase;
- 15           b) regenerating plants from the transformed cell; and
- c) selecting for regenerated plants exhibiting an altered stomatal aperture or altered water-use efficiency or characteristic correlated to same.
- 20           40. The plant cell of claim 36, wherein the polynucleotide corresponds to malic enzyme.
41. The plant cell of claim 36, wherein the construct further comprises a DNA sequence encoding a transit peptide.
- 25           42. The plant cell of claim 41, wherein the transit peptide is a chloroplast transit peptide.
43. A method for increasing productivity in a plant comprising:
- 30           a) transforming a plant cell with a polynucleotide operably linked to a promoter that drives expression in a plant, wherein the polynucleotide is capable of modulating malate accumulation in the plant cell, with the proviso that when the polynucleotide encodes

phosphoenolpyruvate carboxylase, the plant is other than potato or tobacco;

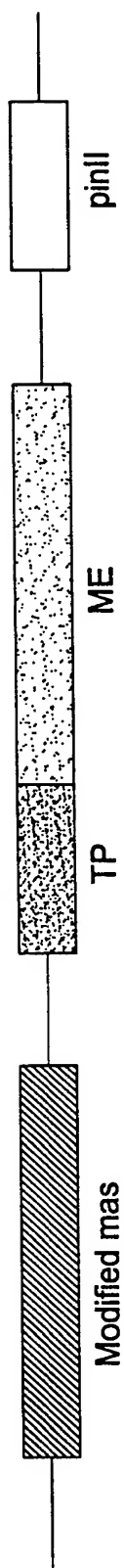
- b) regenerating plants from the transformed cell; and
- c) selecting for regenerated plants exhibiting improved productivity.

5

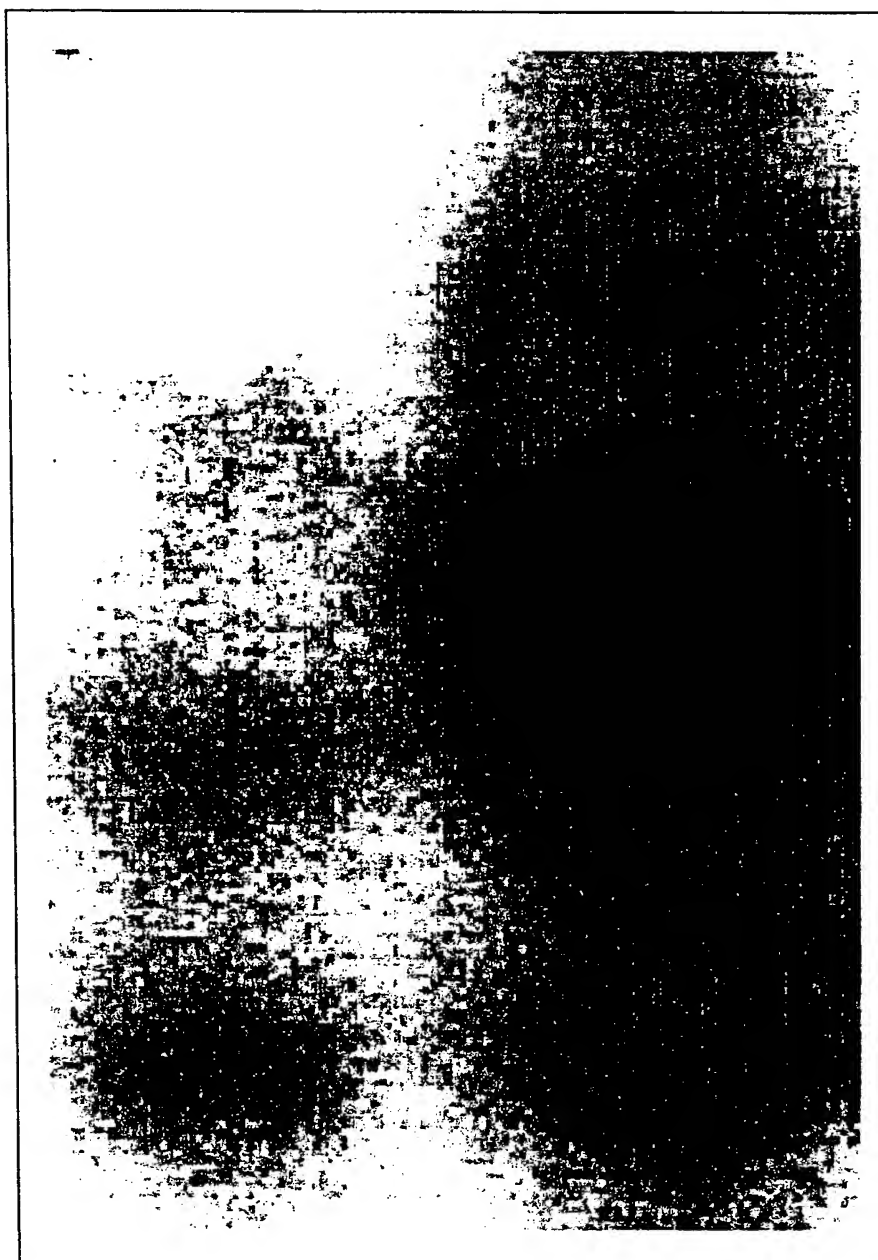
44. The plant of claim 19, wherein the rate of growth of said plant is comparable to the rate of growth of a corresponding wild-type plant.

45. The plant of claim 19, wherein the root-shoot ratio of said plant is  
10 comparable to the root-shoot ratio of a corresponding wild-type plant.

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**FIG. 1.**

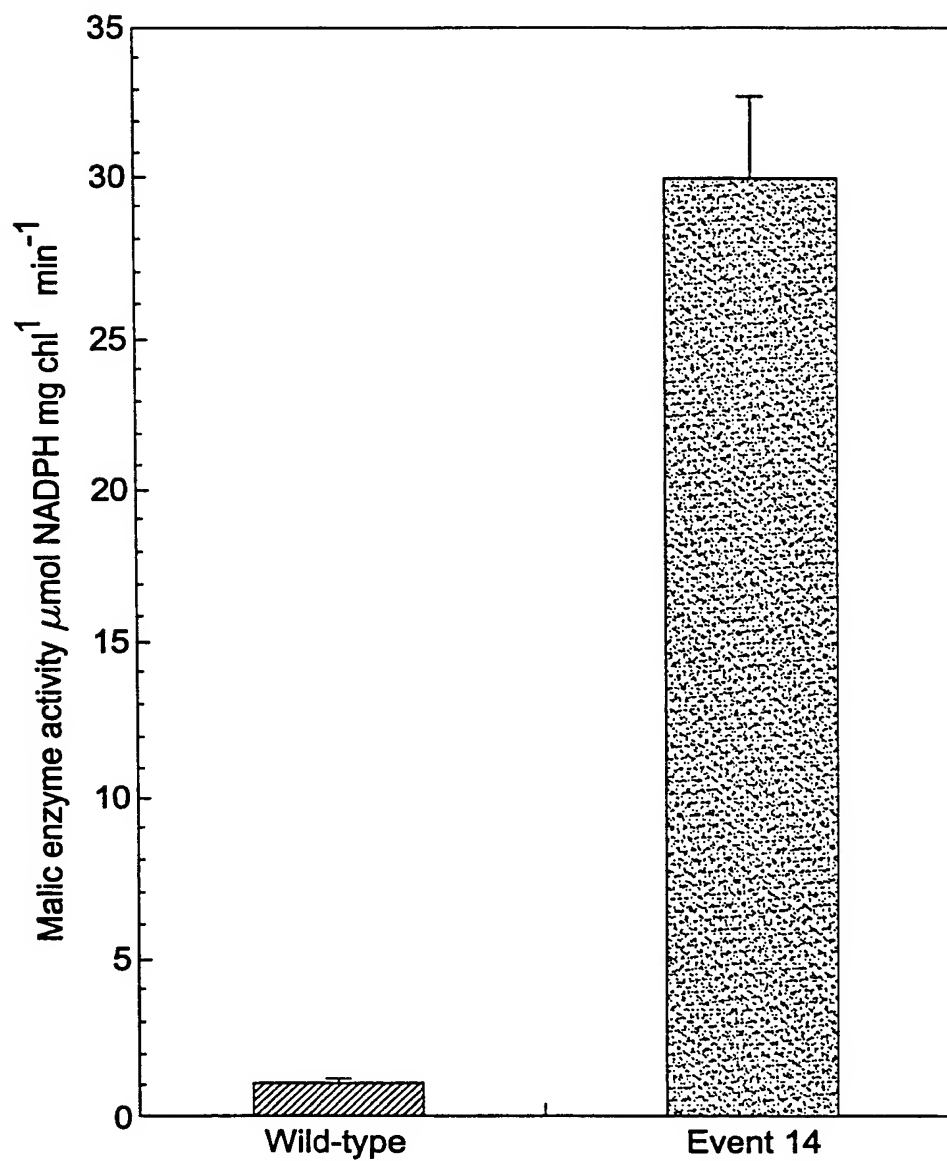


Wild-type

Event 14

FIG. 2A.

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FIG. 2B.



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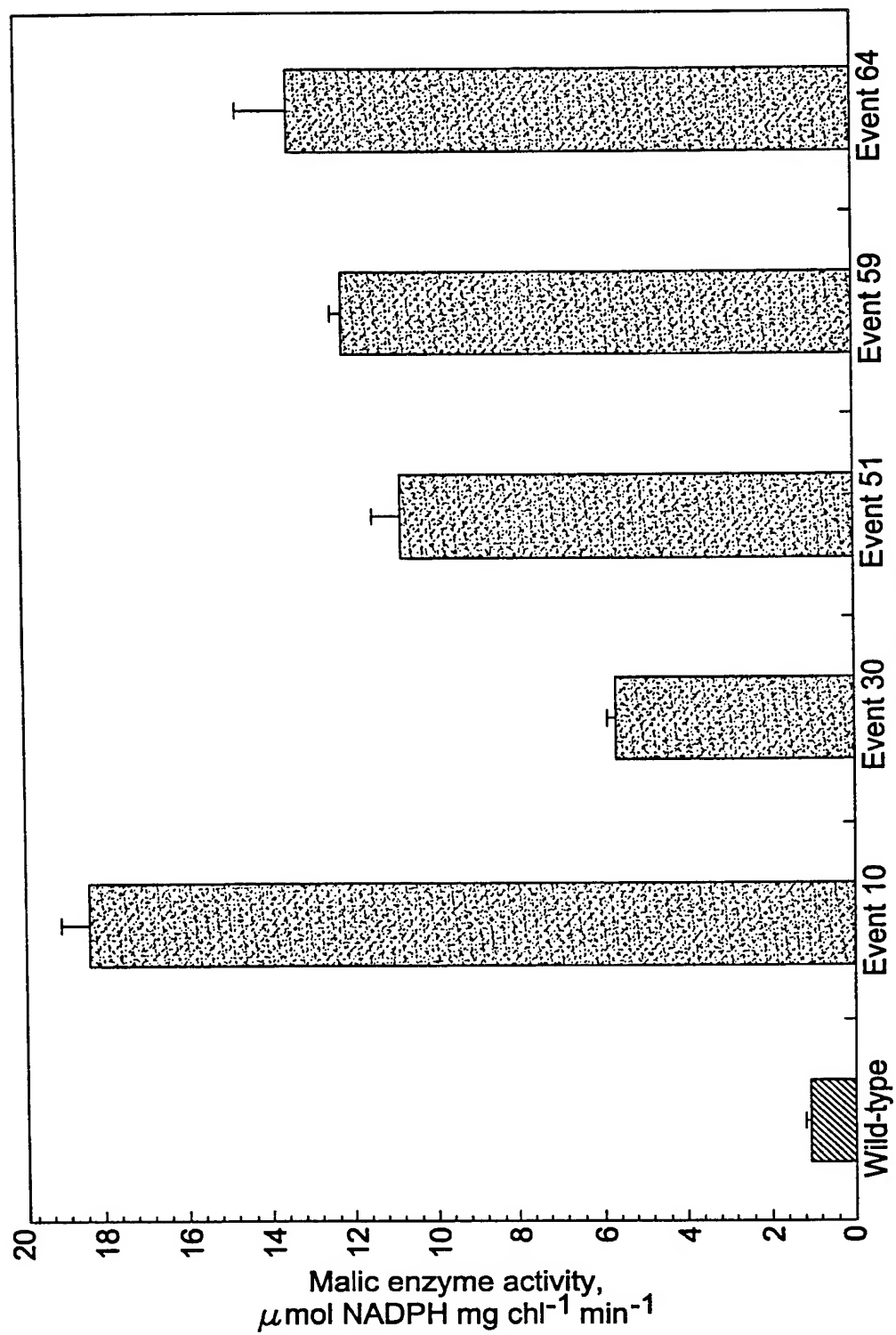


FIG. 3.

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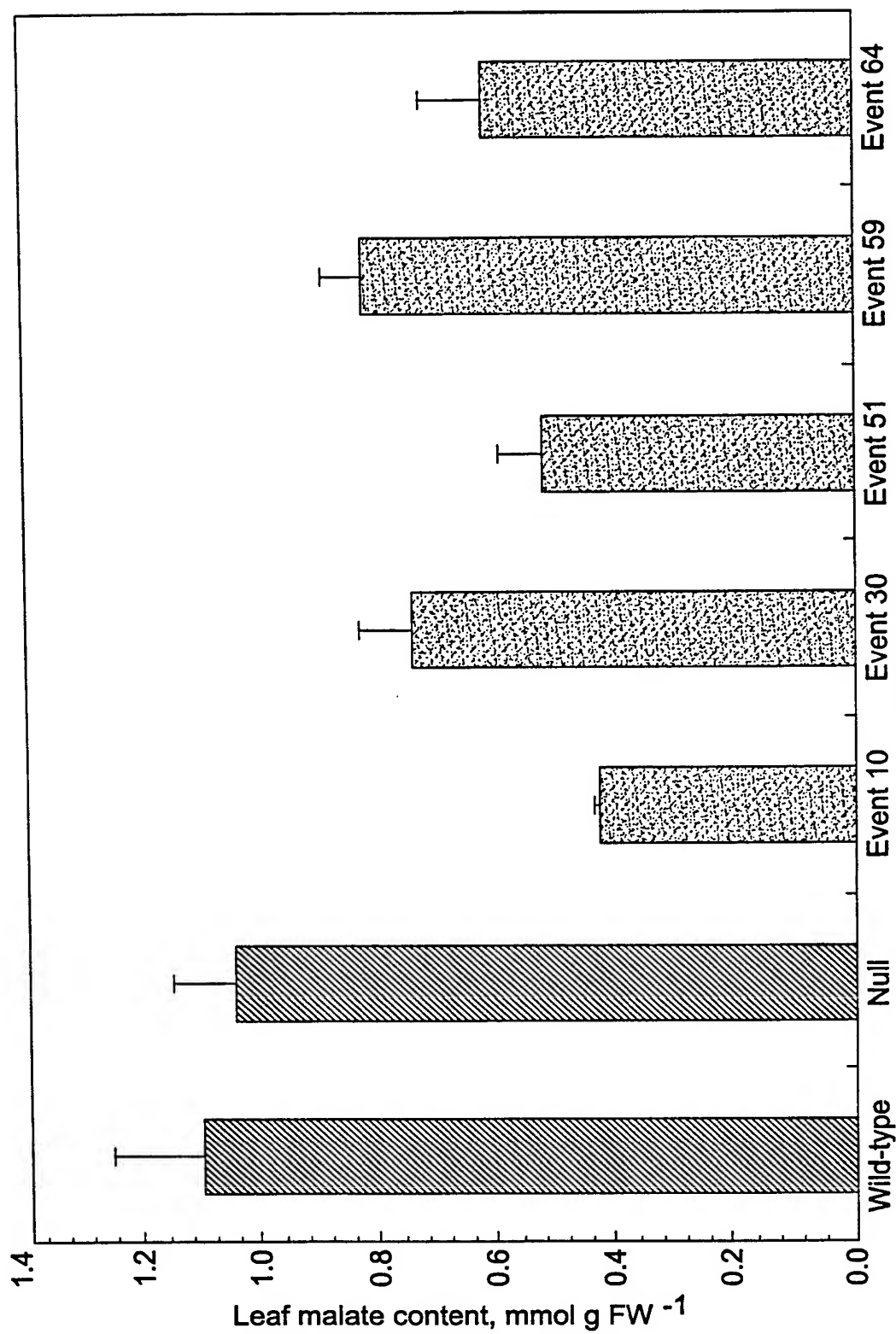
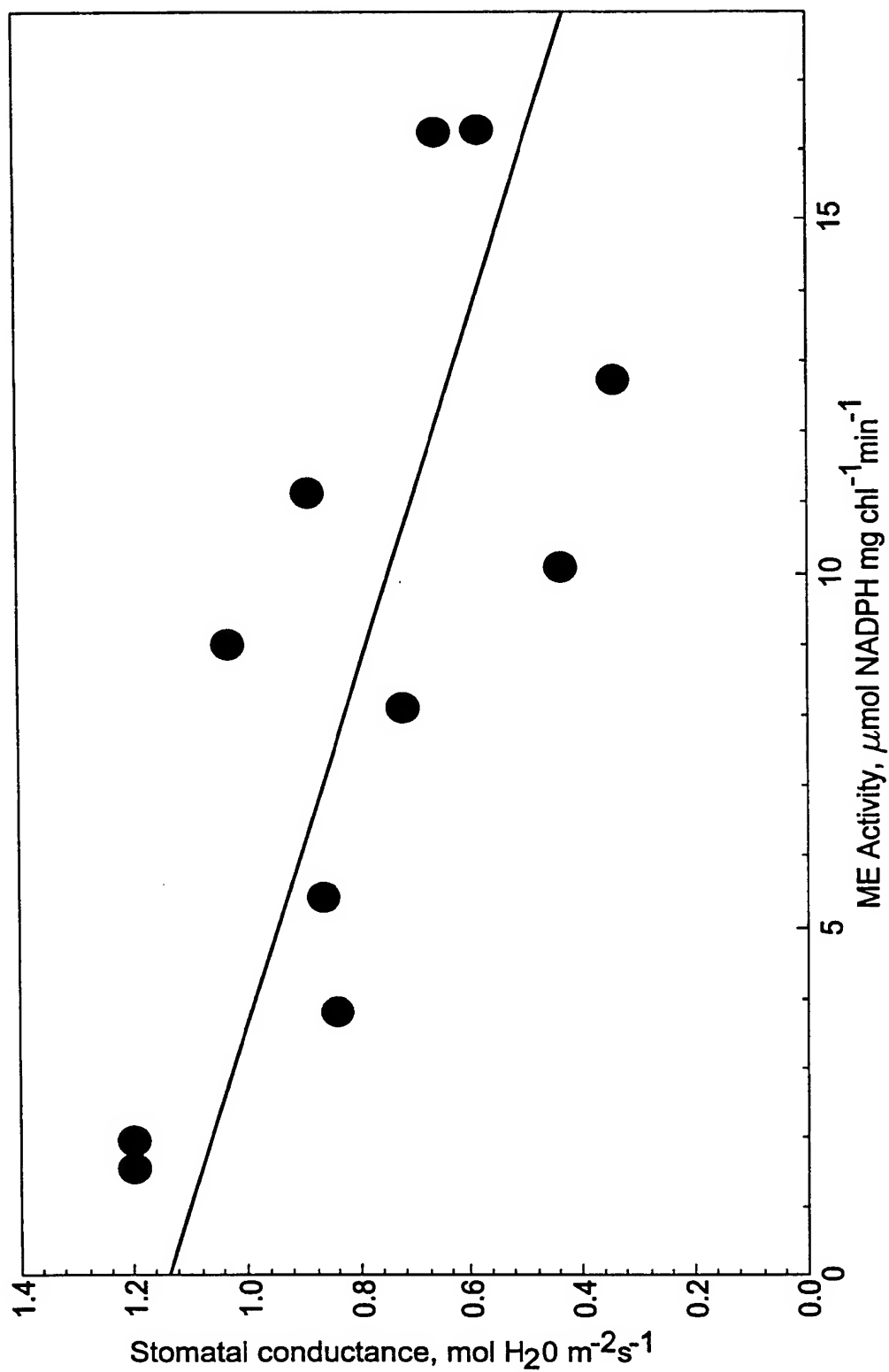


FIG. 4.

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**FIG. 5.**

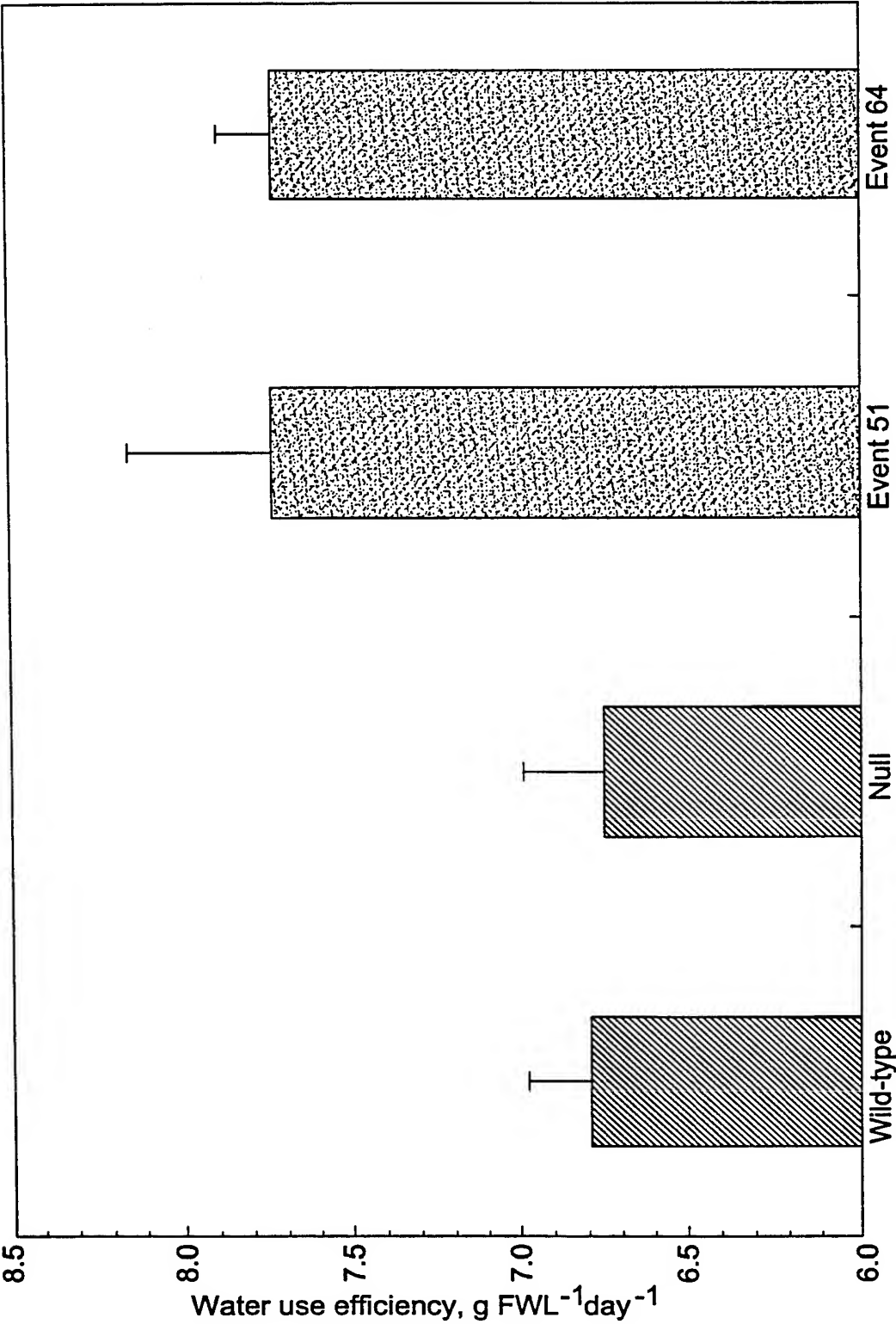


FIG. 6.

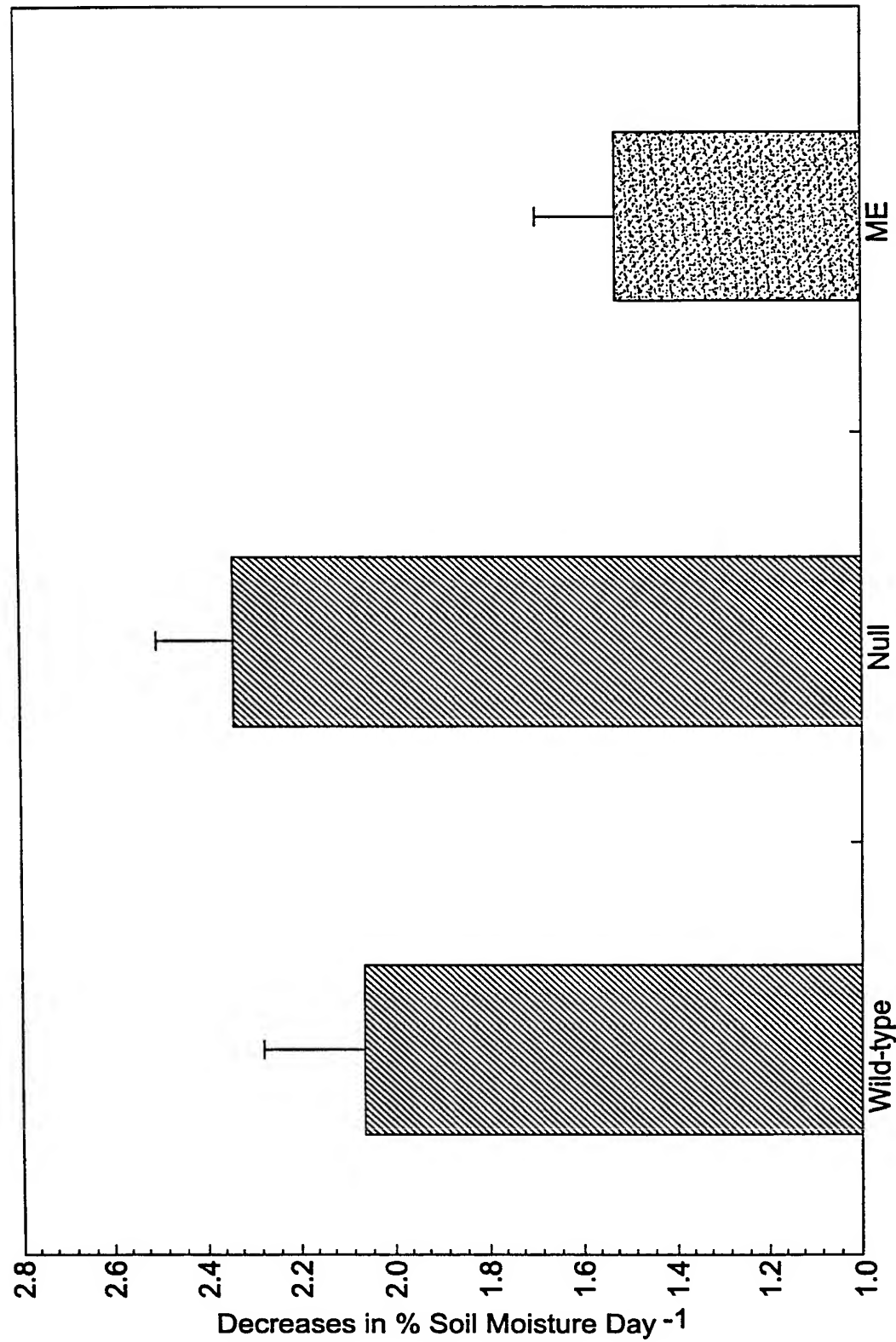


FIG. 7.

## INTERNATIONAL SEARCH REPORT

International Application No.

PCT/US 00/14261

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/82 C12N5/10 A01H5/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EPO-Internal, WPI Data, PAJ

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GEHLEN JOHANNA ET AL: "Effects of altered phosphoenolpyruvate carboxylase activities on transgenic C3 plant <i>Solanum tuberosum</i> ." PLANT MOLECULAR BIOLOGY, vol. 32, no. 5, 1996, pages 831-848, XP002147519	36-38
Y	ISSN: 0167-4412 page 832, right column; page 839, right column; page 840, right column; page 841, 845,846  --- -/--	4-6, 13-15, 36-40

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"&amp;" document member of the same patent family

Date of the actual completion of the international search

15 September 2000

Date of mailing of the international search report

02/10/2000

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Authorized officer

Holtorf, S

## INTERNATIONAL SEARCH REPORT

In tional Application No

PCT/US 00/14261

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KOPKA JOACHIM ET AL: "Potato guard cells respond to drying soil by a complex change in the expression of genes related to carbon metabolism and turgor regulation." PLANT JOURNAL, vol. 11, no. 4, 1997, pages 871-882, XP002147520 ISSN: 0960-7412	1-3,5,6, 10-12, 14,15, 19-31, 36-39, 43-45
Y	abstract, page 872, left column page 879; table 1 ----	4,13,32, 40
A	EP 0 874 056 A (DIRECTOR GENERAL OF NATIONAL I) 28 October 1998 (1998-10-28) the whole document ----	
Y	DATABASE WPI Section Ch, Week 199624 Derwent Publications Ltd., London, GB; Class C06, AN 1996-233343 XP002147524 & JP 08 089250 A (MITSUI TOATSU CHEM INC), 9 April 1996 (1996-04-09) abstract ----	4-6, 13-15, 32,36-40
A	WO 93 18169 A (INST GENBIOLOGISCHE FORSCHUNG) 16 September 1993 (1993-09-16) page 3 -page 5; claim 14 ----	
A	ROTHERMEL B A ET AL: "PRIMARY STRUCTURE OF THE MAIZE NADP-DEPENDENT MALIC ENZYME" JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 264, no. 33, 1989, pages 19587-19592, XP002147521 ISSN: 0021-9258 cited in the application the whole document ----	
A	NI MIN ET AL: "Strength and tissue specificity of chimeric promoters derived from the octopine and mannopine synthase genes." PLANT JOURNAL, vol. 7, no. 4, 1995, pages 661-676, XP002147522 ISSN: 0960-7412 cited in the application the whole document ---- -/--	

## INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 00/14261

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>KOGAMI HIROYUKI ET AL: "Molecular and physiological evaluation of transgenic tobacco plants expressing a maize phosphoenolpyruvate carboxylase gene under the control of the cauliflower mosaic virus 35S promoter." TRANSGENIC RESEARCH, vol. 3, no. 5, 1994, pages 287-296, XP002916978 ISSN: 0962-8819 the whole document -----</p>	



# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 00/14261

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 0874056	A	28-10-1998	JP 10248419 A	22-09-1998
			AU 693754 A	02-07-1998
			CN 1193047 A	16-09-1998
			NZ 329550 A	29-06-1999
JP 8089250	A	09-04-1996	NONE	
WO 9318169	A	16-09-1993	DE 4207358 A	09-09-1993
			AU 671099 B	15-08-1996
			AU 3630493 A	05-10-1993
			CA 2112800 A	16-09-1993
			EP 0584324 A	02-03-1994
			HU 67084 A	30-01-1995
			US 5538879 A	23-07-1996